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ABSTRACT Transgenic corn expressing the insecticidal toxin from *Bacillus thuringiensis* Berliner is gaining support as an effective control technology for use against lepidopteran pests, particularly European corn borer, *Ostrinia nubilalis* Hübner (Lepidoptera: Crambidae). However, there is concern that widespread adoption of transgenic plants will rapidly lead to *B. thuringiensis* toxin resistance. Thus, long-term selection of *O. nubilalis* populations with the Cry1Ab *B. thuringiensis* toxin has been undertaken in several laboratories in the United States and in Europe. We present results from two independent selection experiments performed in laboratories at the University of Nebraska and at the Institut National de la Recherche Agronomique in France. Although the protocols and methods used by the two laboratories were different, the results were comparable. The highest level of resistance occurred at generation 7 (14-fold), generation 9 (13-fold), and generation 9 (32-fold) for three different strains. For each strain, the level of resistance fluctuated from generation to generation, although there were consistently significant decreases in toxin susceptibility across generations for all selected strains. These results suggest that low levels of resistance are common among widely distributed *O. nubilalis* populations.

KEY WORDS *Ostrinia nubilalis*, Lepidoptera, *Bacillus thuringiensis*, resistance, transgenic corn

EUROPEAN CORN BORER, *Ostrinia nubilalis* (Hübner), is a significant lepidopteran pest of maize, *Zea mays* L., in both the United States and Europe. It causes significant economic losses and has been the target of several management strategies (Hudon et al. 1989). Microbial insecticides based on the bacterium *Bacillus thuringiensis* Berliner have been used for managing this pest for many years, although these products have not been widely adopted by growers because of their high cost, narrow spectrum of activity, rapid environmental degradation, and inconsistent control (International Life Sciences Institute 1998). Recently, corn plants that have been genetically transformed to express the Cry1Ab, Cry1Ac, and Cry9C endotoxins from *B. thuringiensis* (referred to as Bt corn) have been developed and are being marketed in the United States and Europe.

Although genetically altered corn plants that produce their own protective pesticides provide an alternative to existing pest control technologies, there is considerable concern that widespread adoption of this technology could result in the development of resistance in pest populations. Since the first report of resistance to a *B. thuringiensis* toxin in Indianmeal moth, *Plodia interpunctella* (Hübner), by McGaughey (1985), resistance has been successfully selected in the laboratory for several insect pest species (for reviews, see Frutos et al. 1999, Sanchis 2000). Diamondback moth, *Plutella xylostella* (L.), is the only insect to evolve high levels of resistance in the field as a result of repeated use of formulated *B. thuringiensis* insecticide (Tabashnik et al. 1990). Field resistance in this species to formulated *B. thuringiensis* insecticides has been reported from the Philippines, Japan, Malaysia, Thailand, Florida, and New York (Tabashnik 1994, Maruyama et al. 1999).

The potential for *B. thuringiensis* resistance development in *O. nubilalis* has prompted numerous studies involving laboratory selections. Resistant strains have been selected from populations collected in Iowa, Kansas, (Huang et al. 1997, 1999a), and southeastern Minnesota (Bolin et al. 1999). The high-dose refuge strategy currently recommended for U.S. corn production (Ostlie et al. 1997, International Life Sciences Institute 1998) requires that the increased fitness conferred by Bt resistance alleles is recessive, i.e., heterozygous individuals either do not survive at doses of toxin produced by Bt corn or survive but with a fitness close to zero (Bourguet et al. 2000). Survival and fitness of heterozygotes depends not only on the dominance of *B. thuringiensis* resistance but also on the resistance level conferred by resistance alleles (Bourguet et al. 2000). Therefore, it is important to determine whether the relatively low resistance ratio reported by Huang et al. (1997, 1999a) and Bolin et al. (1999) is a general feature in *O. nubilalis* populations or whether higher *B. thuringiensis* resistance can be obtained in laboratory-selected populations.

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Long-term selections of *O. nubilalis* populations to the Cry1Ab *B. thuringiensis* toxin have been undertaken in independent laboratories in the United States and in Europe. Herein, we present results from two independent selections performed at the University of Nebraska and at the Unité de Recherches de Lutte biologique at the Institut National de la Recherche Agronomique (INRA) in France. Although the protocols and methods used by the two laboratories were different, the results are comparable and suggest that low levels of resistance in *O. nubilalis* are common.

Materials and Methods

Nebraska Evaluations. *Insects.* Two populations of *O. nubilalis* (Nebraska and Italy) were exposed to laboratory selection. The Italian population (I) was established in 1993 from ≈500 *O. nubilalis* larvae collected in the Lombardia region of northern Italy. The colony was provided to the University of Nebraska after 20 generations of laboratory rearing. The Nebraska colony (N) was established in 1995 from ≈500 field-collected larvae. Field-collected larvae were reared to adults in individual artificial diet (Lewis and Lynch 1969) containers to minimize spread of disease. Adults were mated using standardized rearing techniques. Each population was divided into two subpopulations, one exposed throughout development to Cry1Ab toxin and the other reared in the absence of toxin (control).

Selection and Bioassays. A fermentation paste of *B. thuringiensis* subspecies *kurstaki* strain HD-9 containing ≈9.5% Cry1Ab toxin by weight was used as a source of Cry1Ab for the selection diet (Novartis Seeds, Research Triangle Park NC) and was readily available in large quantities. Selected populations (N and I) were exposed throughout larval development to Cry1Ab toxin incorporated into the artificial diet. The initial concentration of Cry1Ab in the rearing diet was 0.2 μg of active ingredient per ml of rearing diet. The *B. thuringiensis* concentration was gradually increased in later generations to maintain > 70% mortality in the exposed insects. For control strains, five replicates of 300 neonates per rearing container were initiated for each generation and five replicates of 1,000 neonates for selected colonies were initiated for each generation. Rearing containers for selected strains were initiated several days in advance of the control strains to compensate for delayed development associated with sublethal exposure to the Bt toxin and to ensure that neonate larvae from selected and control strains were available for bioassay at the same time. Upon pupation, the total number of pupae in three randomly selected replicates of both control and *B. thuringiensis* selected colonies was counted to estimate percentage of survival from neonate larvae to pupation. Larvae were maintained at 27°C, a photoperiod of 24:0 (L:D) h, and 80% RH. After pupation, surviving insects were transferred to insect mating cages. Adults were maintained at 27°C, a photoperiod of 16:8 (L:D) h and 80% RH. Cages were misted with water twice a day, and adult diet was provided to maximize egg production (Leahy and Andow 1994).

Bioassays for Cry1Ab susceptibility were performed every second generation in 128-well trays (each well 16 mm in diameter by 16 mm in height) as previously described (Marçon et al. 1999). The diet developed for *Heliothis virescens* F. (King et al. 1985) was used in place of rearing diet for bioassays. Three replicates of 16 neonates were exposed to seven different concentrations of Cry1Ab incorporated into the diet (0.002–1.2 μg/ml of diet), and one control concentration without Cry1Ab. The larvae were held at 27°C, a photoperiod of 24:0 (L:D) h, and 80% RH. Individual larval weights were recorded at each toxin concentration 7 d later. Percentage of growth inhibition values were calculated by comparing the mean of treated larval weights to control larval weights. The EC_{50} values (the concentration of Cry1Ab responsible for 50% growth inhibition) and 95% confidence intervals (CI) were determined by nonlinear regression with SAS PROC NLIN and fitted to a probit model with numerical derivatives (SAS Institute 1988) as described by Marçon et al. (1999). An *F* test was used to determine whether the parameters of the nonlinear probit model differed significantly between the control and selected populations.

France Evaluations. *Insects.* Three different populations of *O. nubilalis* were exposed to laboratory selections. Two of the populations were collected from Landes (L) and Alsace (A), two regions of France separated by approximate 1,000 km. The third population was initiated from larvae collected in Switzerland (S) and kindly provided by P. Gay (Novartis, Basel Switzerland). All populations were collected in October 1992 as diapausing larvae, and laboratory rearing was initiated in June 1993. For each site, 150–300 larvae were collected. Each population was divided into two subpopulations, one exposed to the toxin every generation, and the other (control) reared under the same conditions without exposure to the toxin.

Selection and Bioassay. The Cry1Ab toxin was obtained from the 407$^-$sigK (spo$^-$) *cry1Ab Bacillus thuringiensis* strain. This strain was a derivative of the wild-type *B. thuringiensis* strain 407 isolated by Lereclus et al. (1989) which displayed the *cry1Ab* gene described by Sanchis et al. (1988) without producing any spores. Consequently any differences in susceptibility among strains could be attributed to the Cry1Ab toxin. The bacterial strain was grown in a hydrolysate medium supplemented with 0.3% glucose at 30°C, 200 rpm, as previously described (Müller-Cohn et al. 1996). Toxin protein concentrations were measured by the Bradford (1976) method following solubilization with Na_2CO_3 0.5 M, $NaCO_3$ 0.5 M, pH 10.2 (vol:vol), at 37°C, with bovine serum albumin as the standard.

During the first 30 generations of selection, 1,000 neonate larvae (20 replicates of 50 neonates) for each strain (L, A, and S) were exposed individually to concentrations of Cry1Ab spore-crystal preparations spread evenly over the surface of artificial diet.

Table 1. Nonlinear regression of growth inhibition fitted to a probit model for control and Cry1Ab selected *O. nubilalis* populations originating from the Italian collection

Generation	Selected EC_{50} (ng/ml)	95% CL	Control EC_{50} (μg/ml)	95% CL	Resistance ratio at EC_{50}
3	0.0062	0.0053–0.0071	0.0048	0.0041–0.0054	1.29[a]
5	0.0230	0.0037–0.0225	0.0060	0.0001–0.0085	3.83[a]
7	0.0577	0.0326–0.0979	0.0108	0.0080–0.0139	5.34[a]
9	0.0920	0.0221–0.1340	0.0065	0.0057–0.0073	14.10[a]
11	0.0911	0.0395–1.5800	0.0174	0.0001–0.0257	5.24[a]
13	0.0266	0.0213–0.0359	0.0023	0.0012–0.0544	11.30[a]
15	0.0575	0.0296–0.1120	0.0110	0.0175–0.0299	5.22[a]
17	0.0222	0.0130–0.0372	0.0097	0.0080–0.0117	2.28[a]
19	0.0345	0.0208–0.0568	0.0071	0.0060–0.0083	4.86[a]
21	0.0620	0.0252–0.1520	0.0167	0.0115–0.0240	3.70[a]
23	0.0877	0.0449–0.1720	0.0377	0.0322–0.0439	2.32[a]

[a] Significant difference ($P < 0.05$) between control and selected populations based on *F* test to determine differences among parameters of the nonlinear probit model.

Freshly prepared diet was dispensed into wells of 1.65 cm^2 surface area, and individual wells were treated with 25 μl of Bt suspension per well producing >70% mortality after exposure for 5 d. Mortality was recorded after the 5-d exposure and surviving larvae were transferred to untreated artificial diet (Gahukar and Moreau 1976) through pupation. After 30 generations, the L, A, and S strains were combined into a single population referred to as LAS. During the next six generations, insects from this strain were selected individually as described previously. At the seventh generation, a mass selection was initiated with all larvae reared in the same container of treated artificial diet, for 5 d. The dose of Cry1Ab toxin was one μg of protein per cm^2, which caused >90% mortality. Larvae that survived the treatment were transferred to untreated artificial diet and reared individually at 25°C, a photoperiod of 18:6 (L:D) h, and 70% RH. The adults were supplied with honey water for nutrition and paper strips for oviposition.

For each generation, the susceptibility of the three strains to the Cry1Ab toxin was estimated by using bioassays of neonates. Five concentrations of Cry1Ab spore-crystal mixture were applied uniformly over the food surface in each well (25 μl per well) and allowed to dry. A single larva was placed into each well. One replicate of 50 larvae was conducted for each dose. Mortality was recorded after 5 d of exposure. Bioassays were maintained using the same conditions as reported above.

Mortality data were analyzed using the log-probit program of Raymond et al. (1993) based on Finney (1971). This program tests for linearity of dose-mortality curves, provides lethal concentrations, and determines the slope of each dose-mortality line. It also tests the parallelism of two or more dose-mortality lines and computes the resistance ratios at various lethal concentrations with their 95% CL. Resistance ratios were considered to be significantly different from one ($P < 0.05$) when the confidence limit did not include this value.

Results

Nebraska Evaluations. Based on differences in EC_{50} values, the two selected *O. nubilalis* populations showed significantly increased tolerance to the Cry1Ab toxin compared with control strains within the first five to seven generations of exposure (Tables 1 and 2). However, the increased tolerance was not stable, and the resistance ratio fluctuated between 2- and 15-fold in both strains. Highest resistance ratios were observed at generation seven (14-fold) for the N-selected strain and generation nine (13-fold) for the I- selected strain. Estimates of mortality throughout selection experiments and across generations indicated that *O. nubilalis* reared

Table 2. Nonlinear regression of growth inhibition fitted to a probit model for control and Cry1Ab selected *O. nubilalis* populations originating from the Nebraska collection

Generation	Selected EC_{50} (ng/ml)	95% CL	Control EC_{50} (μg/ml)	95% CL	Resistance ratio at EC_{50}
3	0.0246	0.0145–0.0366	0.0134	0.0088–0.0208	1.70
5	0.0231	0.0113–0.0304	0.0143	0.0079–0.0248	1.61
7	0.0800	0.0322–0.2341	0.0055	0.0042–0.0482	14.54[a]
9	0.0555	0.0114–0.1278	0.0209	0.0001–0.0348	2.65[a]
11	0.0114	0.0077–0.0151	0.0067	0.0030–0.0110	1.70
13	0.1005	0.0298–0.0462	0.0164	0.0156–0.0171	6.12[a]
15	0.0180	0.0011–0.0164	0.0076	0.0061–0.0095	2.37[a]
17	0.0210	0.0087–0.0194	0.0081	0.0058–0.0110	2.59[a]
19	0.0691	0.0246–0.1943	0.0151	0.0095–0.0232	4.58[a]
21	0.1240	0.0410–0.3865	0.0291	0.0229–0.0369	4.26[a]
23	0.0600	0.0300–0.1205	0.0176	0.0176–0.0382	3.40[a]

[a] Significant difference ($P < 0.05$) between control and selected populations based on *F* test to determine differences among parameters of the nonlinear probit model.

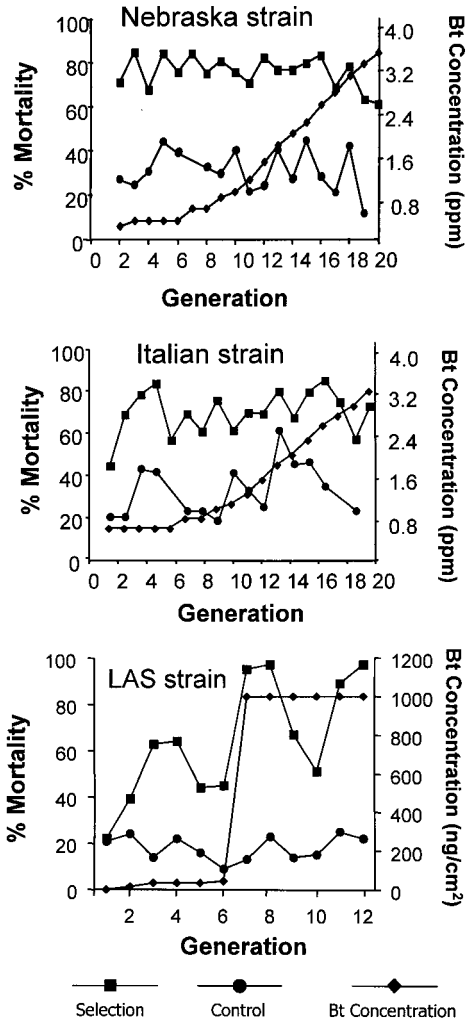

Fig. 1. Comparison of *O. nubilalis* mortality of selected strains on Cry1Ab treated rearing diet versus the control strains maintained on normal rearing diet. For the Nebraska and Italian strains, $N = 900$ neonate larvae for controls and 3,000 for larvae maintained on rearing diet with Cry1Ab incorporated at increasing concenrations; mortality represents the percentage of neonate larvae that did not reach pupation. For the LAS strain, $N > 300$ neonates larvae for controls and >1,000 for selection at each generation; mortality reported is that which occurred within 5-d period of exposure to Cry1Ab applied to the diet surface.

in the presence of *B. thuringiensis* toxin exhibited higher mortality (70–80%) than control strains (20–40%) (Fig. 1). These susceptibility differences between selected and nonselected strains indicate that selection pressure was being applied to both the N and I strains although the level of selection was lower than anticipated because of relatively low survival in the unselected controls. Additionally, the I-selection displayed significantly elevated tolerance to the Cry1Ab toxin when reared in the absence of toxin for one generation (data not shown).

France Evaluations. After 30 generations of selection, the three initial strains (L, A, and S) did not exhibit a significant level of resistance to the Cry1Ab toxin (data not shown). The three strains were then combined into a single LAS strain and further selected for another 12 generations. Fig. 1 outlines the details of the selection pressures for the LAS strain and the associated mortality during the 12 generations of selection. *O. nubilalis* selected in the presence of the *B. thuringiensis* toxin exhibited higher mortality (39–97%) compared with the untreated strain (10–25%). After two generations of selection, the LC_{50} of the selected LAS strain was five-fold higher than the control strain and was significantly greater than 1 (Table 3). From the fourth generation and until the last generation of selection, the selected LAS strain was consistently and significantly ($P < 0.05$) less susceptible than the control strain. As previously described for the selected strains obtained at the University of Nebraska, the resistance ratio did not steadily increase but fluctuated between 2- and 32-fold at the seventh and 32nd generation, respectively.

Discussion

All selected strains of *O. nubilalis* developed significantly increased tolerance after chronic exposure to the Cry1Ab toxin. These results support those of Huang et al. (1997) and Bolin et al. (1999) who also observed significantly decreased susceptibility of *O. nubilalis* populations after exposure to *B. thuringiensis* toxins for multiple generations. In populations from Iowa and Kansas (Huang et al. 1997), significant resistance was found after three to seven generations of exposure to Dipel ES, a composite of Cry1Aa, Cry1Ab, Cry1Ac, Cry2A, and Cry2B endotoxins and after four generations of selection with Cry1Ac toxin in Minnesota populations (Bolin et al. 1999). Two of the initial subpopulations selected at the University of Nebraska did not develop significant levels of resistance despite continued exposure for at least 10 generations (data not shown), suggesting that there is some variation in the ability of populations to develop resistance.

The levels of resistance obtained in our strains were smaller than those reported by Huang et al. (1997) (73-fold) and by Bolin et al. (1999) (162-fold). The highest level of resistance occurred at generation 7 (14-fold), generation 9 (13-fold), and generation 9 (32-fold) for the N, I, and LAS strains, respectively. For each strain, susceptibility fluctuated from generation to generation, although there were consistently elevated EC_{50} and LC_{50} across generations observed for all selected strains. These results indicate the chronic exposure to the Cry1Ab toxin throughout development did select for increased *B. thuringiensis* tolerance. When Bolin et al. (1999) switched from surface applications of the toxin to incorporation of the toxin in the diet, the 162-fold change in susceptibility fell to 16- to 56-fold over the next 13 generations. This offers a partial explanation for the slightly higher resistance ratio observed in the LAS strain (selected with surface applications) compared with those of the Nebraska and Italian strains (selected with incorporated toxin). Surface applications may also explain the higher generation to generation variability recorded

Table 3. Lethal concentrations for control and Cry1Ab selected (LAS) *O. nubilalis* strains originating from a combination of strains L and A (France) and S (Switzerland)

Generation	Selected LC_{50} ($\mu g/cm^2$)	95% CL	Control LC_{50} ($\mu g/cm^2$)	95% CL	Resistance ratio at LC_{50} (95% CL)
1	0.02	0.05–0.07	0.02	0.01–0.02	1 (0.9–2)
2	0.31	0.16–1.53	0.06	0.04–0.12	5 (3–8)[a]
3	0.09	0.02–0.31	0.04	0.02–0.10	2 (0.7–6)
4	0.18	0.11–0.36	0.02	0.01–0.08	8 (4–16)[a]
5	0.80	0.37–5.48	0.03	0.02–0.04	8 (22–69)[a]
6	0.96	0.42–15.18	0.12	0.10–1.68	8 (3–18)[a]
7	0.16	0.07–0.28	0.08	0.05–0.13	2 (1.5–2.5)[a]
8	2.81	1.66–4.77	0.26	0.13–0.54	11 (7–16)[a]
9	10.07	3.37–30.23	0.31	0.22–0.45	32 (16–66)[a]
10	1.35	0.69–2.21	0.17	0.06–0.48	8 (4–17)[a]
11	0.64	0.28–1.42	0.07	0.03–0.13	11 (6–20)[a]
12	0.31	0.10–0.53	0.02	0.01–0.02	19 (12–31)[a]

The L, A and S strains were individually selected using Cry1Ab during 30 generations without a significant change in susceptibility.
[a] LC_{50} of selected strain is significantly ($P < 0.05$) different from LC_{50} of nonselected (control) strain.

for the LAS strain. Another source of variation in resistance ratios is the absence of replicates of the bioassay performed each generation on the LAS strain. Indeed, despite efforts to control experimental error, significant variation could have occurred in the preparation and implementation of each bioassay run.

Variation in the susceptibility to Cry1Ab toxin may also be the result of a contribution of multiple genes with relatively small resistance effects. A synergistic effect of several genes is supported by the results obtained from the French evaluations. The three original strains (L, A and S) were selected for resistance for 30 generations without showing any decrease in Cry1Ab susceptibility. When these strains were combined, the resulting strain (LAS) did show response to selection. While the primary concern for Bt resistance development in the field is the presence of rare major resistance alleles, the multiple effect of minor and/or modifier genes may still contribute to fitness in the field.

Bacillus thuringiensis resistance has been described in Lepidoptera and Coleoptera, suggesting that the potential to evolve resistance to *B. thuringiensis* is widespread among target insect species (Tabashnik 1994, Frutos et al. 1999, Sanchis 2000). The mechanisms of decreased susceptibility of *O. nubilalis* are still relatively unclear. Huang et al. (1999b) characterized the midgut proteinases from susceptible and resistant strains of European corn borer. In one of these strains, the hydrolyzing efficiency of trypsin-like proteinases decreased 35% compared with the susceptible strain, indicating a reduced activation of the *B. thuringiensis* protoxin. However, no significant difference was found in the three other resistant strains.

The founding population size was small (500 individuals) for all the populations we examined. Similarly, the populations selected by Huang et al. (1997) and Bolin et al. (1999) were founded from 200–300 larvae and 32 mated pairs, respectively. Such low initial genetic diversity is likely to intensify during laboratory rearing and sampling bottlenecks before selection. Because the founding populations were small, our results indicate that partial resistance to *B. thuringiensis* toxins must be relatively common among natural populations of *O. nubilalis.* Results obtained from the F_2 screen described by Andow and Alstad (1998) have provided additional support for the inference that partial resistance alleles to *B. thuringiensis* toxins are prevalent in natural populations of *O. nubilalis.* In three different F_2 screens performed in 1996 and 1997, the 95% CI for the frequency of partial resistance were $[1 \times 10^{-3}, 1.5 \times 10^{-2}]$ for a Minnesota population (Andow et al. 1998, Andow and Alstad 1999) and $[8.2 \times 10^{-4}, 9.4 \times 10^{-3}]$ for an Iowa population (Andow et al. 2000). An additional F_2 screen has confirmed such a high frequency for partial resistance alleles in an *O. nubilalis* population sampled in southern France and the U.S. northern corn belt (Bourguet et al. unpublished data).

Conversely, alleles that confer high levels of resistance appear to be rare. Andow et al. (1998, 2000) and Andow and Alstad (1999) estimated such alleles to be rare in Minnesota (<0.009 with 95% CI) and Iowa (<3.9 times 10^{-3} with 95% CI) populations of *O. nubilalis* based on results of the F_2 screen. Therefore, it is not surprising that alleles that confer high levels of resistance were absent in the populations of *O. nubilalis* used to initiate the laboratory selections that have been performed thus far. Hence, although the selection regimes used in these experiments provide evidence of genetic adaptation, the results may not reflect the potential resistance that might develop when field populations are exposed to transgenic plants. Indeed, it is unlikely that these selected populations could survive on transgenic Bt corn, although such tests remain to be conducted. Thus, these results should be interpreted with caution.

Resistance management models, such as the high-dose/refuge (Alstad and Andow 1995, Gould 1998, International Life Sciences Institute 1998) are considered as the best approach for managing transgenic crops and are currently being recommended for Bt corn in North America (Ostlie et al. 1997). Initially, the high-dose/refuge concept was motivated by resistance to *B. thuringiensis* toxins in other species being recessive, and by high expression levels of *B. thurin-*

giensis toxins in plants ensuring that heterozygotes could not survive on Bt crops (Alstad and Andow 1995, Gould 1998, Roush 1998). However, the survival of heterozygous individuals depends not only on the dominance level but also on the resistance ratio and the doses expressed by transgenic Bt crops (Bourguet et al. 2000). Thus, even an incompletely dominant *B. thuringiensis* resistance (Huang et al. 1999a) could give a recessive fitness at *B. thuringiensis* concentrations expressed by transgenic Bt corn (Bourguet et al. 2000). Furthermore, heterozygote survival may be influenced by variability in Bt expression among tissues, by the amount of resources available to the developing larva, and by larval density on host plants all of which contribute to variation in toxin exposure over space and time.

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